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BONE MORPHOGENETIC PROTEIN 2 STIMULATES ARTICULAR CARTILAGE PROTEOGLYCAN SYNTHESIS IN VIVO BUT DOES NOT COUNTERACT INTERLEUKIN-1α EFFECTS ON PROTEOGLYCAN SYNTHESIS AND CONTENT

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Objective. **To study the effect of bone morphogenetic protein 2 (BMP-2) on articular cartilage proteoglycan (PG) synthesis in vivo and to investigate whether BMP-2 is able to counteract the effects of interleukin-1 (IL-1) on articular cartilage PG synthesis and content.**

Methods. **BMP-2 alone or in combination with IL-1α was injected into murine knee joints. PG synthesis was measured by ^{35}S-sulfate incorporation using an ex vivo method or autoradiography. Cartilage PG content was analyzed by measuring Safranin O staining intensity on histologic sections.**

Results. **BMP-2 appeared to be a potent stimulator of articular cartilage PG synthesis in vivo. However, BMP-2 was not able to counteract the deleterious effects of IL-1α on articular cartilage PG synthesis and content. In addition, intraarticular injections of BMP-2 induced chondrophytes.**

Conclusion. **Although BMP-2 is a very potent stimulator of cartilage PG synthesis in vivo, the therapeutic applications of BMP-2 are limited due to the inability of BMP-2 to counteract the effects of IL-1 and the induction of chondrophytes.**

Rheumatoid arthritis (RA) is a disease characterized by chronic inflammation of the joints. The disease causes cartilage degradation, which results in the loss of joint function. An early event in the process of cartilage degradation is depletion of proteoglycans (PG) from articular cartilage. Interleukin 1 (IL-1) is an important mediator in this process. IL-1 is able to enhance PG degradation and to suppress PG synthesis (1–4). Moreover, studies in which IL-1 was neutralized during experimental arthritides demonstrated that IL-1 is directly involved in the inhibition of articular cartilage PG synthesis (4–7).

Factors which are able to counteract the effects of IL-1 on chondrocyte metabolism or which are able to stimulate the replenishment of PGs in the depleted matrix could be of significant therapeutic value. In this respect, bone morphogenetic proteins (BMPs) seem promising. BMPs belong to the transforming growth factor β (TGFβ) superfamily (8,9). This superfamily consist of dimeric molecules, each monomer of which contains 7 conserved cysteine residues (10). The proteins signal by serine/threonine kinases (8,11).

One of the members of the BMP family with potential therapeutic value is bone morphogenetic protein 2 (BMP-2). BMP-2 has been demonstrated to be a potent stimulator of chondrocyte metabolism and differentiation (12–15). BMP-2 is a potent stimulator of PG synthesis by articular cartilage explants in vitro (12), but until now, no in vivo data about the effects of BMP-2 on articular cartilage PG synthesis have been available. Neither have any data been published about the ability of BMP-2 to counteract the effects of IL-1 on articular cartilage. We therefore studied the effect of BMP-2 on murine articular cartilage PG synthesis in vivo and investigated the ability of BMP-2 to counteract the effects of IL-1 on articular cartilage PG synthesis and content.

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MATERIALS AND METHODS

Animals. Male C57Bl/6 mice between ages 8 and 12 weeks were used. Mice were fed a standard diet and tap water ad libitum.

Growth factors and cytokines. Recombinant murine IL-1α (0.48 mg/ml in phosphate buffered saline, pH 7.4) and recombinant human TGFβ1 (0.1 mg/ml in 20 m*M* NaOAc, pH 5.0) were kindly provided by Pfizer Central Research (Groton, CT) and Genentech (South San Francisco, CA), respectively. Recombinant human BMP-2 (2.27 mg/ml in 0.5*M* arginine, 10 m*M* histidine, pH 6.5) was supplied by Genetics Institute (Cambridge, MA). To prevent loss of protein due to adherence to plastic, only siliconized tubes and tips were used.

Intraarticular injections. To study the effect of BMP-2 on articular cartilage PG synthesis in vivo, 6 μl of physiologic saline plus 0.1% bovine serum albumin including recombinant human BMP-2 (2–1,000 ng) was injected into the joint cavity of the right knee. The ability of BMP-2 to counteract the effects of IL-1 on articular cartilage PG synthesis and content was studied by injecting IL-1α (10 ng), either alone or in the presence of BMP-2 (200–1,000 ng). The dose of 10 ng IL-1α has been demonstrated to suppress articular cartilage PG synthesis after a single injection and to induce significant PG depletion in multiple injection protocols (2). A single injection was given, or 3 injections were given on alternate days. Since we have previously demonstrated that TGFβ1 is able to counteract the effects of IL-1 on articular cartilage PG synthesis and content (16,17), we used coinjections of IL-1α (10 ng) and TGFβ1 (200 ng) as positive controls.

Histology. Whole knee joints were dissected and fixed for 7 days in phosphate buffered formalin. The fixed knee joints were decalcified (5% formic acid) and dehydrated by an automated tissue processing apparatus (VIP; Miles Scientific, Naperville, IL). After embedding in paraffin wax, semiserial frontal knee sections (6 μm) were prepared and mounted on gelatin-coated slides. Paraffin was removed by xylol and ethanol, and sections were stained with Safranin O and fast green (18). Safranin O staining, a semiquantitative marker of PG depletion, was measured using an automated image analysis system (VIDAS; Kontron Electronics, Munich, Germany) (19). Fast green staining was neutralized by use of a green filter. Optical density was examined in the noncalcified cartilage of the patella. Measurements were corrected for chondrocyte lacunae. Staining values were corrected for background staining, as measured in PG-depleted patellar cartilage in which red stain was no longer visible. PG depletion was induced by intraarticular injection of papain (19). Each experimental group contained at least 8 knee joints, of which 3 semiserial sections were analyzed.

Autoradiographic analysis of ^{35}S-sulfate incorporation was performed as described (18). Radiolabeled sulfate (75 μCi) was injected intraperitoneally 6 hours before dissection of the knee joints. After histologic processing, 6-μm sections were prepared and mounted on gelatin-coated slides. These were dipped in K_5 emulsion (Ilford Basildon, Essex, UK) and exposed for 3 or 5 weeks. After this period, the slides were developed and stained with hematoxylin and eosin.

Determination of patellar cartilage PG synthesis. Proteoglycan synthesis was measured ex vivo according to the method of van den Berg et al (20). Whole patellae were

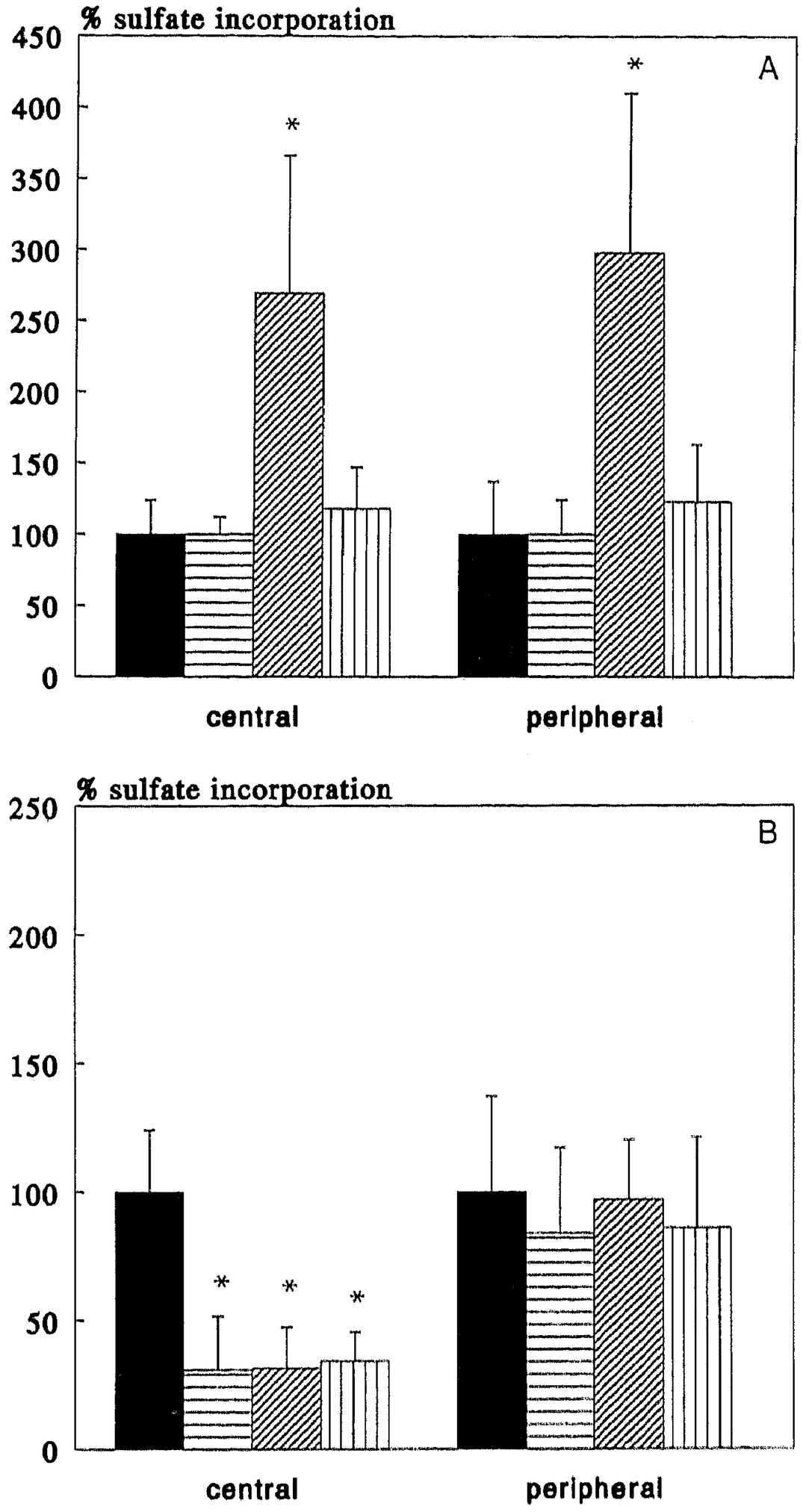

Figure 1. Patellar cartilage synthesis of proteoglycans in untreated knees (solid bars) and in knees on day 2 after a single intraarticular injection of vehicle (horizontal-striped bars), bone morphogenetic protein 2 (200 ng) (hatched bars), or transforming growth factor β1 (200 ng) (vertical striped bars) with (**A**) or without (**B**) coinjection of 10 ng interleukin-1α. ^{35}S-sulfate incorporation (mean ± SD percentage of incorporation in untreated knees; n = 6) was measured after an ex vivo pulse-labeling of isolated patellae with ^{35}S-sulfate. ^{35}S-sulfate incorporation by central and peripheral areas of untreated knees was comparable (400–700 cmp). * = $P \leq 0.05$ versus vehicle-injected knees, by Student's *t*-test. (Note: y-axis scales differ in **A** and **B**.)

dissected from the knee joints and pulse-labeled (3 hours at 37°C) with ^{35}S-sulfate (30 μCi/ml). Subsequently, they were washed, fixed in ethanol, and decalcified in formic acid. After decalcification of the patellae, the entire cartilage was stripped off, and a 0.2 mm^2 round section was punched out of the center (central part) that remained in the peripheral area (21). The central and peripheral areas of the patellar cartilage were dissolved and ^{35}S-incorporation was counted by liquid scintillation counting. Each experimental group contained at least 6 patellae.

RESULTS

Effect of BMP-2 on articular cartilage PG synthesis in vivo. Intraarticular injection of BMP-2 (2–1,000 ng) resulted in stimulation of patellar cartilage PG synthesis, which was maximal 2 days after injection. The effect of BMP-2 on PG synthesis was dose dependent, with a maximum stimulation of 250% at a dosage of ≥200 ng (data not shown). To investigate whether BMP-2 has differential effects on PG synthesis in the central and peripheral areas of the patella, PG synthesis of both areas was measured separately.

BMP-2 stimulated patellar cartilage PG synthesis by both the central and peripheral areas equally (Figure 1A). On day 2 after intraarticular injection of TGFβ1 (200 ng), no significant effect on articular cartilage PG synthesis was noted (Figure 1A).

Absence of counteraction by BMP-2 of IL-1α-induced PG synthesis inhibition. Since BMP-2 appeared to be a potent stimulator of patellar cartilage PG synthesis, we evaluated whether BMP-2 was able to counteract the suppressive effect of IL-1 on cartilage PG synthesis. IL-1α (10 ng) was injected into murine knee joints in the presence or absence of 200 ng of BMP-2. Two days after intraarticular injection of IL-1α, total patellar PG synthesis was ~40% lower than in controls. Suppression of PG synthesis by IL-1α alone was higher in the central part (mean ± SD 69 ± 20%) than in the peripheral part (16 ± 33%) (Figure 1B). Simultaneous injection of 10 ng IL-1α and 200 ng BMP-2 resulted in suppression of PG synthesis to a degree similar to that induced by IL-1α alone (Figure 1B). Coinjection with higher concentrations of BMP-2 (up to 1 μg) had no effect on IL-1α–induced suppression (data not shown).

These results demonstrate that although BMP-2 is a potent stimulator of articular cartilage PG synthesis, it is unable to counteract the inhibition of articular cartilage PG synthesis induced by IL-1α. Moreover, these results also indicate that the effects of BMP-2 on cartilage PG synthesis are blocked when chondrocyte metabolism is affected by IL-1α. In parallel with BMP-2,

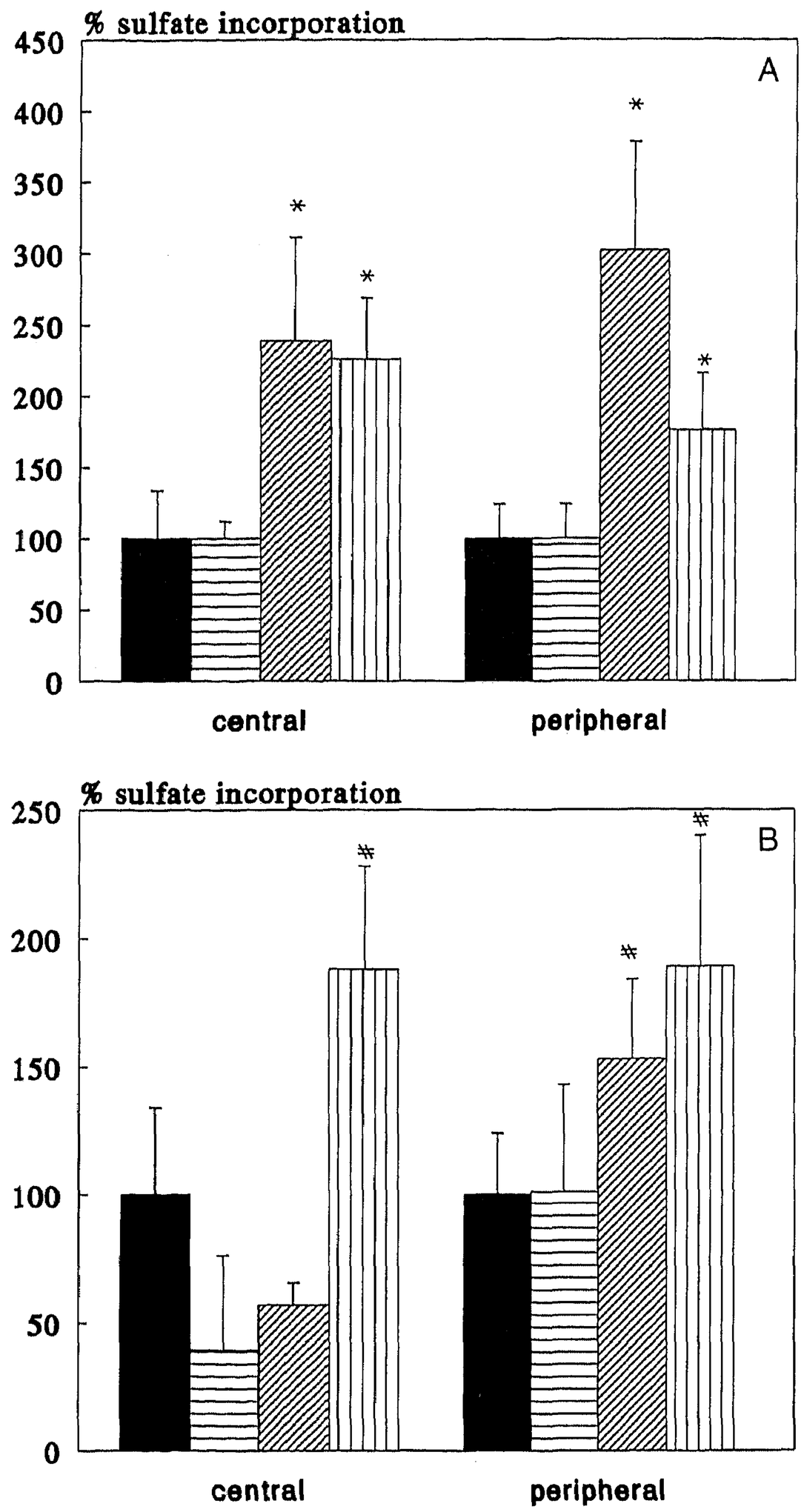

Figure 2. Patellar cartilage synthesis of proteoglycans in untreated knees (solid bars) and in knees on day 2 after 3 intraarticular injections of vehicle (horizontal-striped bars), bone morphogenetic protein 2 (200 ng) (hatched bars), or transforming growth factor β1 (200 ng) (vertical striped bars) with (**A**) or without (**B**) coinjection of 10 ng interleukin-1α. (IL-1α). ^{35}S-sulfate incorporation was measured as described in Figure 1, and expressed as the mean ± SD percentage of untreated knees (n = 6). ^{35}S-sulfate incorporation by central and peripheral areas of untreated knees was comparable (400–700 cmp). * = $P < 0.05$ versus vehicle-injected knee joints; # = $P < 0.05$ versus IL-1α–injected knees, by Student's *t*-test. (Note: y-axis scales differ in **A** and **B**).

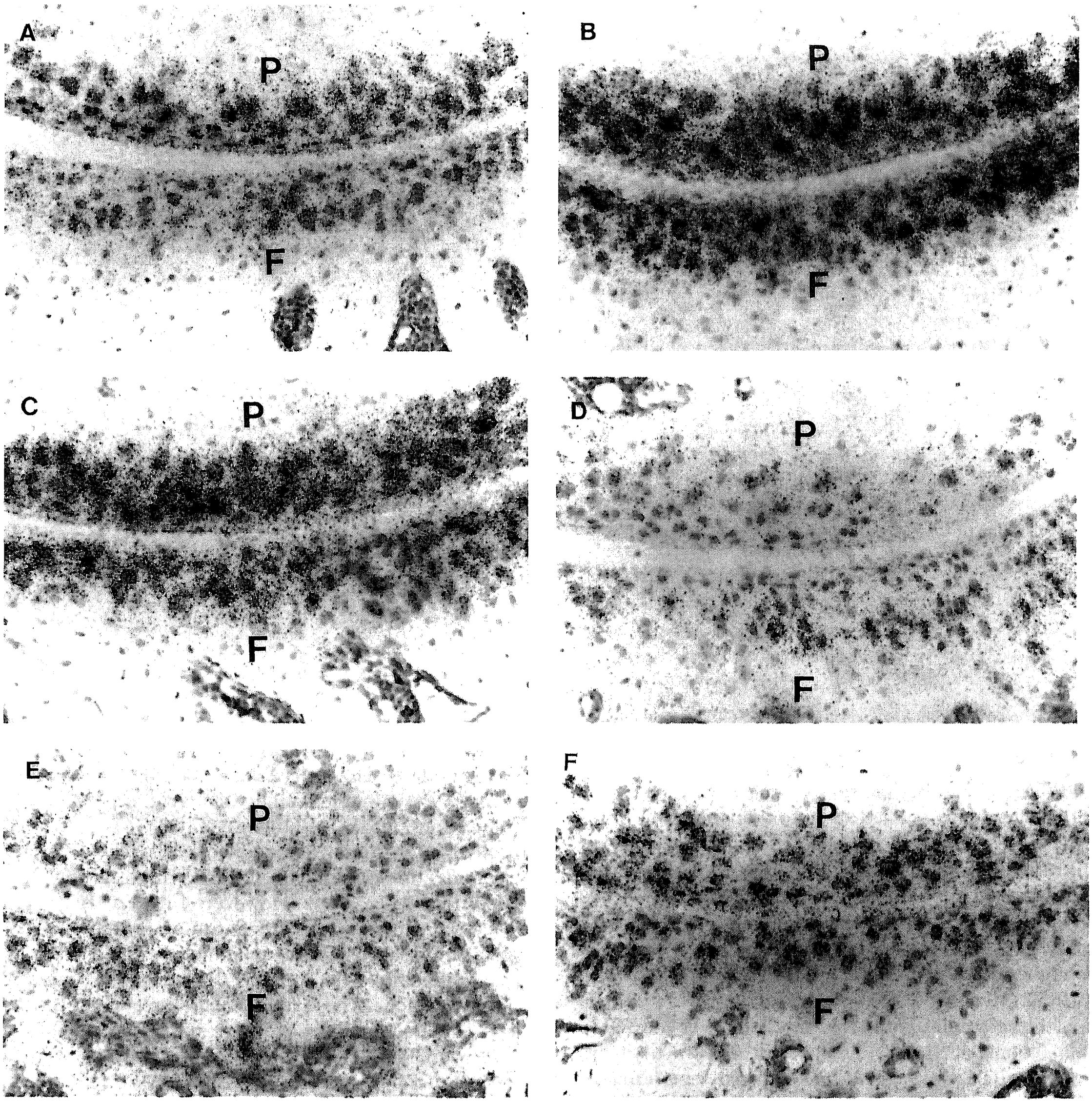

Figure 3. Autoradiographs showing ^{35}S-sulfate incorporation in the central regions of patellar and femoral cartilage on day 1 after 3 intraarticular injections of **A,** vehicle, **B,** bone morphogenetic protein 2 (BMP-2) (200 ng), **C,** transforming growth factor β1 (TGFβ1) (200 ng), **D,** interleukin-1α (IL-1α) (10 ng), **E,** IL-1α (10 ng) + BMP-2 (200 ng), and **F,** IL-1α (10 ng) + TGFβ1 (200 ng). ^{35}S-sulfate was injected intraperitoneally 6 hours before dissection of the knee joints. After histologic processing, autoradiography was performed. (Original magnification × 200.) **P** = patella; **F** = femur.

coinjection with TGFβ1 (200 ng) demonstrated no effect on IL-1α–induced suppression of PG synthesis on day 2 after injection (Figure 1B).

Effect of long-term BMP-2 exposure on IL-1α–induced inhibition of PG synthesis. In previous studies, we showed that long-term exposure of articular cartilage

to TGFβ1 resulted in stimulation of articular cartilage PG synthesis, while short-term exposure had no effect on PG synthesis (22). We sought to determine whether long-term, rather than short-term, exposure of cartilage to BMP-2 could counter IL-1α–induced suppression of PG synthesis. We therefore gave 3 coinjections on alternate days. BMP-2 (200 ng) and TGFβ1 (200 ng) stimulated articular cartilage PG synthesis in both the central and peripheral areas of the patellar cartilage (Figure 2A). Coinjections of IL-1α (10 ng) and BMP-2 (200 ng) resulted in strong suppression (>60%) of PG synthesis in the central part of the patella, which was not significantly different from the synthesis after 3 injections with IL-1α alone (Figure 2B). This indicates that long-term exposure of cartilage to BMP-2 is also not able to counteract the effects of IL-1α.

In contrast to the central region, PG synthesis in the peripheral region in the mice injected with IL-1α alone was not different from that of the controls. Knee joints injected with IL-1α (10 ng) and BMP-2 (200 ng) showed a significant stimulation of PG synthesis in the peripheral area of the patella. In contrast to BMP-2, injections of TGFβ1 (200 ng) in the presence of IL-1α (10 ng) resulted in stimulation of PG synthesis in both the central and peripheral regions of the patellar cartilage (Figure 2B), demonstrating that TGFβ1 is able to counteract IL-1α–induced suppression of articular cartilage PG synthesis after long-term exposure.

Autoradiographic analysis of local effects of BMP-2 on PG synthesis. To investigate the effects of BMP-2 on chondrocytes from various sites of the joint, PG synthesis was studied by autoradiography of histologic sections. Figure 3 shows autoradiographs of the central regions of the patellar cartilage and the facing femoral cartilage 1 day after 3 injections. BMP-2 (200 ng) stimulated PG synthesis in femoral cartilage to a similar extent as in patellar cartilage and homogeneously throughout the articular cartilage (Figure 3B). Injections of IL-1α (10 ng) resulted in suppression of PG synthesis in both the patellar and femoral cartilage (Figure 3D). Coinjections of IL-1α (10 ng) and BMP-2 (200 ng) suppressed PG synthesis in patellar and femoral cartilage to a similar extent as injections of IL-1α alone (Figure 3E), demonstrating that BMP-2 did not counteract the IL-1α–induced inhibition of PG synthesis in either the patellar or the femoral cartilage. In contrast, coinjections of IL-1α (10 ng) and TGFβ1 (200 ng) resulted in stimulation of PG synthesis in both the patellar and femoral cartilage (Figure 3F), as compared with injections of IL-1α alone (Figure 3D). Similar effects were demonstrated in the femorotibial joint (not shown).

Table 1. Safranin O staining of histologic sections of patellar cartilage on day 1 or day 4 after triple intraarticular injections*

Injection	% staining intensity	
	Day 1	Day 4
Vehicle	100 ± 12	100 ± 16
Interleukin-1α (10 ng)	69 ± 12†	76 ± 11†
+ BMP-2 (200 ng)	71 ± 12†	77 ± 22†
+ TGFβ1 (200 ng)	67 ± 14†	107 ± 21‡

* Whole knee joints were dissected 1 or 4 days after triple intraarticular injections. Histologic sections of patellar cartilage were stained with Safranin O and quantified using an automated image analyzer. Values were corrected for background staining, as measured in completely depleted patellar cartilage. Each group contains at least 8 knee joints, from which 3 histologic sections were analyzed. BMP-2 = bone morphogenetic protein 2; TGFβ1 = transforming growth factor β1.
† $P < 0.05$ versus vehicle-injected knee joints, by Student's *t*-test.
‡ $P < 0.05$ versus interleukin-1α–injected knee joints, by Student's *t*-test.

Effect of BMP-2 on IL-1α–induced PG depletion. Changes in patellar cartilage PG content were measured after triple injections with IL-1α alone or in combination with BMP-2 or TGFβ1. The PG content of articular cartilage is reflected in the intensity of Safranin O staining of histologic sections. As shown in Table 1, significant loss of Safranin O staining was noted on day 1 and day 4 after 3 injections of 10 ng IL-1α. Injections with IL-1α (10 ng) in the presence of 200 ng BMP-2 demonstrated that BMP-2 had no effect on patellar cartilage PG content neither on day 1 nor on day 4. Coinjections of IL-1α (10 ng) and TGFβ1 (200 ng) resulted in an initial depletion of PGs (day 1 after 3 injections) that was indistinguishable from the depletion induced by IL-1α alone. However, 4 days after the last injection, patellar cartilage Safranin O staining was significantly more intense in joints that had been injected with both IL-1α and TGFβ1 than in those injected with IL-1α alone. These results demonstrate that BMP-2 does not modify IL-1α–induced PG depletion and, in contrast to TGFβ1, is not able to accelerate the replenishment of PG in the depleted matrix.

Induction of chondrophytes by BMP-2. Histologic sections of the knee joints demonstrated that intraarticular injection of BMP-2 (200 ng) resulted in the formation of new chondroid tissues (Figure 4B). These chondrophytes were predominately localized in the patellofemoral area. Chondrophytes were also induced after coinjections of IL-1α (10 ng) and BMP-2 (200 ng) (Figure 4D). This indicates that although IL-1α blocks

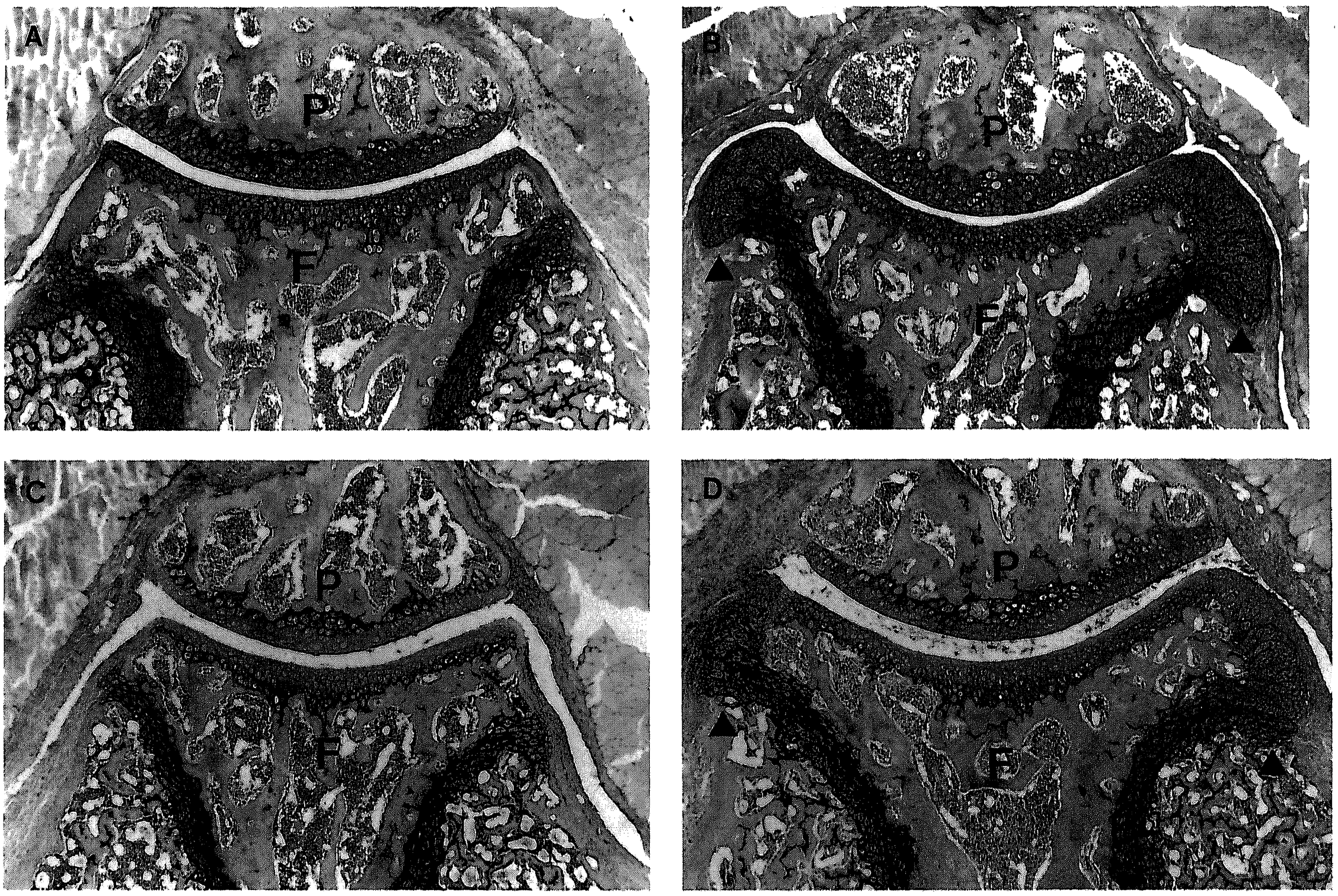

Figure 4. Histologic features of knee joint sections stained with Safranin O, showing the induction of chondrophytes by bone morphogenetic protein 2 (BMP-2) either in the absence or presence of interleukin-1 (IL-1). Whole knee joints were dissected 1 day after triple intraarticular injections of **A,** vehicle, **B,** BMP-2 (200 ng), **C,** IL-1α (10 ng), and **D,** IL-1α (10 ng) + BMP-2 (200 ng). (Original magnification × 100.) **P** = patella; **F** = femur; **arrows** show chondrophytes.

the effects of BMP-2 on articular cartilage PG synthesis, it does not block the induction of chondrophytes by BMP-2.

DISCUSSION

Factors that are able to counteract the deleterious effects of IL-1 on articular cartilage PG synthesis and content or that have the potential to accelerate the replenishment of PGs in depleted cartilage can be expected to be of significant therapeutic value. Because BMP-2 has been demonstrated to be a potent regulator of chondrocyte metabolism and differentiation (12–15), we studied the ability of BMP-2 to stimulate articular cartilage PG synthesis and to counteract the effects of IL-1 on articular cartilage PG synthesis and content in vivo.

This is the first study in which the in vivo effects of BMP-2 on articular cartilage PG synthesis are described. BMP-2 appeared to be a potent stimulator of articular cartilage PG synthesis in vivo. PG synthesis was stimulated homogeneously throughout the articular cartilage. In vitro, members of the BMP family (BMP-2, BMP-3, BMP-4, and BMP-7) have been demonstrated to stimulate articular chondrocyte PG synthesis (12,23,24), but until now, there were no in vivo data. Also, the BMP-2–related factor TGFβ1 was, as we described before (16,22,25), a potent stimulator of articular cartilage PG synthesis in vivo. Interestingly, the effect of BMP-2 and TGFβ1 on PG synthesis shows different kinetics. For instance, 1 and 2 days after intraarticular injection of BMP-2, articular cartilage PG synthesis was significantly stimulated, whereas injection of TGFβ1 did not significantly affect PG synthesis before the third day

after injection. The difference in kinetics between BMP-2 and TGFβ1 indicates that in vivo, chondrocytes respond immediately to BMP-2, but the TGFβ1 responses are dependent on a second mediator or on changes in chondrocyte reactivity to TGFβ1 induced by the injected TGFβ1 itself.

Although BMP-2 stimulated articular cartilage PG synthesis in vivo, it was unable to counteract the suppression of articular cartilage PG synthesis that was induced by 10 ng of IL-1α. No significant effect of BMP-2 could be demonstrated in cartilage showing IL-1–induced inhibition of PG synthesis. However, it cannot presently be completely excluded that BMP-2 is able to counteract the effects of lower concentrations of IL-1α. Other members of the BMP family have been reported to inhibit IL-1–induced suppression of articular cartilage PG synthesis in vitro. For example, BMP-7 counteracted the effect of IL-1α on chondrocyte PG synthesis in bovine cartilage explants (24). However, no data on the in vivo effects of BMPs on IL-1–induced suppression of articular cartilage PG synthesis have been published until now.

The lack of change in articular chondrocyte PG synthesis induced by BMP-2 in the presence of IL-1α indicates that the effect of BMP-2 on articular cartilage PG synthesis is blocked when chondrocyte metabolism is affected by IL-1α. The mechanism of IL-1–induced BMP-2 nonresponsiveness is presently unclear. Down-regulation of BMP-2 receptors or blocking of intracellular signaling pathways by IL-1 are 2 possibilities. In contrast to BMP-2, TGFβ1 was able to counteract IL-1–induced suppression of articular cartilage PG synthesis, indicating that chondrocytes that are affected by IL-1α still have the capability to react to TGFβ. These results indicate that IL-1α obstructs the BMP-2, but not the TGFβ1, signaling pathway in articular chondrocytes.

Members of the BMP family seem to have, at least in vitro, the potential to inhibit the synthesis of PG-degrading enzymes and to inhibit PG breakdown itself. BMP-7 suppressed IL-1–induced up-regulation of collagenase (MMP-1) and stromelysin (MMP-3) messenger RNA and counteracted the IL-1–induced inhibition of their natural inhibitor (TIMP) (26). BMP-3 and BMP-4 have been shown to inhibit PG degradation in cartilage explants cultured in vitro (23). We therefore examined the effects of BMP-2 on IL-1–induced PG depletion in articular cartilage. Safranin O staining of patellar cartilage on histologic sections demonstrated that BMP-2 did not affect IL-1α–induced PG depletion. Although TGFβ1 appears to be an inhibitor of the catabolic effects of IL-1 on articular cartilage in vitro (27–29), it was unable to inhibit IL-1–induced PG depletion in vivo. The discrepancy between in vitro and in vivo findings could be attributed to mediators produced by synovial cells or by inflammatory cells that are attracted to the joint by coinjections of TGFβ1 and IL-1α (16).

The ability of BMP-2 to accelerate the replenishment of PGs in IL-1–depleted cartilage was studied by measuring Safranin O staining intensity on day 4 after 3 injections with IL-1α. We demonstrated that BMP-2 did not enhance restoration of PG content in IL-1–depleted matrix at this time. In contrast to BMP-2, TGFβ1 clearly stimulated repair in the depleted matrix. The different effects of BMP-2 and TGFβ1 on the replenishment of PGs in the depleted matrix can be explained by the different abilities of BMP-2 and TGFβ1 to counteract the IL-1–induced suppression of articular cartilage PG synthesis.

Intraarticular injections of BMP-2 resulted in the formation of new chondroid tissue, especially in the patellofemoral area. As we demonstrated previously, intraarticular injections of TGFβ1 also induced chondrophytes (22). Interestingly, BMP-2–induced chondrophytes are quite different from TGFβ1-induced chondrophytes (Van Beuningen et al: unpublished observations). The ability of BMP-2 to induce the formation of new cartilage and bone has been demonstrated by the rat ectopic bone formation assay (9,30,31). Other members of the BMP family, such as BMP-3, BMP-4, BMP-5, and BMP-7, are also able to induce new cartilage and bone in vivo (30–33). Although IL-1α appeared to block the effects of BMP-2 on articular cartilage PG synthesis, the formation of chondrophytes was not inhibited by IL-1α, which demonstrates that IL-1 does not block all BMP-2–mediated responses in the joint.

In summary, this study demonstrates that BMP-2 is a potent stimulator of articular cartilage PG synthesis. However, when chondrocyte metabolism is affected by IL-1α, the stimulatory effect of BMP-2 on PG synthesis is completely blocked. Because IL-1 is present in arthritic joints (34–37), our results indicate that BMP-2 alone cannot be used to stimulate cartilage repair during arthritis. Although BMP-2 appears to be unable to stimulate cartilage repair when IL-1 is present, BMP-2 might stimulate cartilage repair in the presence of IL-1 inhibitors such as IL-1 receptor antagonist. In addition, BMP-2 could have the potential to stimulate cartilage repair in pathologic conditions, such as cartilage trauma, in which it is unlikely that IL-1 is involved. However, formation of chondrophytes might limit the therapeutic applications of BMP-2.

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